VIROLOGY 217, 582–593 (1996)
ARTICLE NO. 0153

Enhanced Infectivity of Modified Bluetongue Virus Particles for Two Insect Cell Lines

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Received June 26, 1995; accepted January 19, 1996

Previous studies (Mertens *et al., Virology* 157, 375–386, 1987) have shown that removal of the outer capsid layer from bluetongue virus (BTV) significantly reduces (approximately $\times 10^{-4}$) the infectivity of the resultant core particle for mammalian cells (BHK 21 cells). In contrast, the studies reported here, using a cell line (KC cells) derived from a species of *Culicoides* that can act as a vector for BTV (*Culicoides variipennis*), demonstrated a much higher infectivity of core particles than that in mammalian cells (approximately $\times 10^{3}$). This increase resulted in a specific infectivity for cores that was only 20-fold less than that of purified disaggregated virus particles (stored in the presence of 0.1% sodium-*N*-lauroylsarcosine (NLS)). Removal of this detergent caused intact virus particle aggregation and (as previously reported) resulted in an approximately 1 $\log_{10}$ drop in the specific infectivity of those virus particles which remained in suspension. In consequence the specific infectivity of core particles for the KC cells was directly comparable to that of the intact but aggregated virus. These data are compared with the results from oral infectivity studies using two vector species (*C. variipennis* and *Culicoides nubeculosus*), which showed similar infection rates at comparable concentrations of purified cores, or of the intact but aggregated virus particles (NLS was toxic to adult flies). The role of the outer core proteins (VP7) in cell attachment and penetration, as an alternative route of initiation of infection, is discussed. Previous studies (Mertens *et al., Virology* 157, 375–386, 1987) also showed that the outer capsid layer of BTV can be modified by proteases (including trypsin or chymotrypsin), thereby generating infectious subviral particles (ISVP). The specific infectivity of ISVP for mammalian cells (BHK21 cells) was shown to be similar to that of disaggregated virus particles. In contrast, we report a significantly higher specific infectivity of ISVP but not of the intact virus (approximately $\times$ 100) for two insect cell lines (KC cells and C6/36 mosquito cells (derived from *Aedes albopictus*)). In oral infection studies with adults of the two vector species, ISVP produced the same infection rate at approximately 100-fold lower concentrations than either core particles or the intact but aggregated virus particles. The importance of mammalian host serum proteases, or insect gut proteases, in modification of the intact virus particle to form ISVP and their role in initiation of infection and the vector status of the insect is discussed.

INTRODUCTION

Bluetongue virus (BTV) is the type species of the *Orbivirus* genus within the family Reoviridae (Holmes *et al.,* 1995). BTV causes an arthropod-borne infectious disease of ruminants. The only known biological vectors of BTV are species of biting midge of the genus *Culicoides.* Of more than 1000 *Culicoides* species only 17 have been positively connected with BTV and of these only 6 species (*Culicoides imicola, Culicoides variipennis, Culicoides fulvus, Culicoides wadai, Culicoides actoni,* and *Culicoides nubeculosus*) have been found to transmit the virus (reviewed by Mellor, 1990). In nonvector species and in refractory individuals within a vector species, virus in the lumen of the midgut is unable to initiate a systemic infection of the host. Although the basis of this insusceptibility is not known, it has been suggested that it may be due to a "gut barrier" phenomenon. This could involve virus inactivation by digestive fluids, impermeability of the peritrophic membrane, a surface-type defence mechanism, variations in the permeability of the gut cell membranes for virus, the availability of suitable receptor sites for virus particles on the cell surface, an inability of the virus to replicate in the insect gut cells, or if replication can occur in these cells, a failure to release virus from the abluminal surface of the gut cells into the insect haemocoel (Chamberlain and Sudia, 1961). It is also possible that modification of virus particles by digestive fluids may alter the "state" of the virus particles themselves and could result in an increase, rather than a decrease, in their ability to infect the cells of the gut wall. A requirement for and the occurrence of such modification could therefore also play a significant role in initiation of infection and consequently in any gut barrier effect.

At least three different BTV particle types have been identified which contain genomic dsRNA segments and which have been characterised in terms of their structural protein components. These include intact virus particles, infectious subviral particles (ISVP), and cores (Verwoerd *et al.,* 1972; Mertens *et al.,* 1987). BTV core particles contain two major structural proteins

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0042-6822/96 $18.00

(VP3 and VP7) and three distinct minor proteins (VP1, VP4, and VP6/VP6a) surrounding the 10 dsRNA genome segments and have active RNA polymerase and capping enzyme activities. In whole virus particles, the core is surrounded by an outer capsid layer containing two additional major structural proteins (VP2 and VP5). At least five nonstructural proteins are also synthesised during BTV replication in mammalian cells (which include proteins NS1, NS1a, NS2, NS3, and NS3a). NS2 can become associated with the outer capsid layer and is present in virus particles purified by the techniques used for this study (Mertens *et al.,* 1984, 1987). ISVP are produced by treatment of intact BTV virus particles with a range of proteolytic enzymes (including trypsin or chymotrypsin) which results in loss of protein NS2 and cleavage of VP2 (the major neutralising antigen and haemagglutinin) into a number of smaller polypeptides which remain associated with the outer capsid layer (Mertens *et al.,* 1987, 1989). ISVP have a similar specific infectivity to intact, nonaggregated virus particles for mammalian cells (BHK 21 cells). In contrast, the BTV core particles produced by uncoating of virus particles or ISVP *in vitro* using an appropriate cation treatment have entirely lost the outer capsid proteins (VP2, VP5, and NS2) and have a very much reduced ($\times 10^{-4}$) infectivity for mammalian cells (Mertens *et al.,* 1987).

The presence of chymotrypsin and of several other proteases has been confirmed in the midgut of a wide variety of haematophagous insects, ranging from tsetse flies and muscids to mosquitoes and sandflies (Houseman, 1980; Champlain and Fisk, 1956; Akov, 1972; Briegal and Lea, 1975; Gooding, 1972; Thomas and Gooding, 1972; Spiro-Kern and Chen, 1972), although no information is currently available concerning the nature of the proteases present in *Culicoides* species.

With other members of the Reoviridae the state of the outer capsid layer of the virus particle and in particular its complete removal, or modification by proteolytic enzymes, can not only change the specific infectivity of the virus but also its mode of entry into the host cell (Borsa *et al.,* 1979; Reddy and Black, 1977; Elias, 1977; Nuss, 1983; Reddy and MacLeod, 1976; Zarbl and Millward, 1983; Sharpe and Fields, 1983; Holmes, 1983; Francki and Boccardo, 1983; Payne and Mertens, 1983; Joklik, 1983; Tosteson *et al.,* 1993). The retention, or increase in infectivity of reoviruses, rotaviruses, or cypoviruses (occluded within polyhedra) in the environment of the host gut is essential for infection of the host to occur via this route. Plant reoviruses and orbiviruses are transmitted between their plant or mammalian hosts (respectively) by the bite of insect vectors. The ability of these viruses to remain infectious in the environment of the gut lumen is likely to be irrelevant during infection of their definitive hosts but is an essential requirement for oral infection of the insect vector species. The duration and level of particle infectivity within the gut could therefore play an important part in determining the efficiency of vector transmission.

The aims of these studies were to assess the ability of virus particles, ISVP, and cores of BTV to infect mammalian and insect cells, by measurement of their specific infectivity for BHK21 cells and for two cell lines, derived from a vector and nonvector species (*C. variipennis* and *Aedes albopictus,* respectively). The susceptibility of whole insects to infection by arboviruses can be defined both in terms of the minimum virus titre necessary to initiate a productive and persistent infection and by the percentage of insects in a given population which become infected (susceptibility rate) (Mellor, 1990). The minimum particle number required to initiate infection in any individuals, and the persistent infection rates produced by ingestion of the different BTV particle types as part of a blood meal, were assessed in adults of *C. variipennis* and *C. nubeculosus* (a highly susceptible and a relatively less susceptible vector species, respectively). The potential significance of the different particle types and the role of particle modification within the insect gut during the initial stages of infection are discussed.

MATERIALS AND METHODS

Insects

Adult female *C. variipennis* and *C. nubeculosus* from the colonies maintained at the IAH, Pirbright, were used in these experiments (Boorman, 1974). The *C. variipennis* colony was established in 1967 using eggs supplied by Dr. H. Jones in Denver, Colorado. The *C. nubeculosus* colony was established in 1969 from pupae collected in the wild from Hertfordshire, England.

Viruses and calculation of particle numbers

BTV serotype 1 from South Africa (BTV1 SA) was originally obtained from the Veterinary Research Institute, Onderstepoort, South Africa. BTV serotype 4 (Asot 1) was obtained as a sheep isolate from the 1969 bluetongue outbreak in Cyprus (Parker *et al.,* 1975). BTV serotype 3 was obtained as an isolate from an infected sheep (15th June 1944) in Cyprus. These viruses were plaque purified three times and are identical to those described previously (Sangar and Mertens, 1983; Mertens *et al.,* 1984, 1987, 1989; Pedley *et al.,* 1988). Virus, ISVP, and cores of BTV1 SA, BTV3, and BTV4, either unlabelled or labelled with [^{35}S]methionine, were grown in BHK21 cells and purified as described by Mertens *et al.* (1987). The purified particles were usually dialysed against 0.1 *M* Tris/HCl, pH 8.0, and stored at 4°. Virus particles and ISVP of BTV1 SA and BTV3 are unstable in CsCl solutions and were therefore purified in sucrose gradients. Some of these preparations (as indicated), containing sucrose but not the detergent sodium-*N*-lauroylsarcosine (NLS)

(which was used during purification and to prevent virus particle aggregation, Mertens *et al.,* 1987) were stored at 4° and used for oral infectivity studies without dialysis, thereby avoiding the resultant increase in volume and reduction in particle concentration. The specific infectivity of intact virus or ISVP dropped by less than 1 $\log_{10}$, after storage for 1 year at 4°. However, the infectivity of the core particles showed a significant and progressive decline and they were therefore used for the infectivity studies described here within 2 weeks of purification.

The numbers of particles per A_{260} unit, for each of the intact virus, core, and ISVP preparations used in these experiments, were estimated from total molecular weights for virus particles and cores of BTV1 SA, of 10.8×10^7 and 6.7×10^7, which contain an estimated 88 and 80.5% protein, respectively. These values were calculated from the total molecular weight of the BTV1 SA genome of 13.1×10^6 (A. M. Wade-Evans, personal communication), the total number of VP7 copies per particle (780) (Grimes *et al.,* 1995), and the ratios of the structural proteins (including NS2) in purified particles (Burroughs *et al.,* 1995 and Burroughs, unpublished data). Mertens *et al.* (1987) reported that preparations of virus and cores of BTV serotype 1, purified by the methods used here, contain 400 and 200 μg of protein per A_{260} unit, respectively (using a "Biorad" protein estimation kit). These values are equivalent to absorbance/particle ratios of 1.0 A_{260} unit of cores $= 2.2 \times 10^{12}$ particles and 1.0 A_{260} unit of intact virus $= 2.5 \times 10^{12}$ particles. Because of the similarity of the protein and RNA composition of ISVP and virus particles (Mertens *et al.,* 1987) it is estimated that preparations of ISVP will also contain approximately 2.5×10^{12} particles per A_{260} unit. These values are comparable to those previously calculated for reovirus of 2.1×10^{12} per A_{260} unit (Joklik, 1983; Smith *et al.,* 1969).

Estimates of particle numbers were also made by electron microscopy. Preparations of purified particles (10 μl) were mixed with equal volumes containing latex beads (86 ± 6 nm diameter; Agar Scientific; 1.43×10^{12}/ml final concentration). One microliter of this mixture was added to 4 μl of 0.1% BSA and dried onto formvar/carbon-coated EM grids, previously treated with poly-L-lysine. The grids were stained with 2% KOH-buffered PTA and examined in a Jeol 1200 EX microscope. The original number of virus particles in each preparation was determined from the ratio of viral particles to latex beads observed and compared to their optical density measured at 260 nm. Using these methods it was estimated that one A_{260} unit of cores contained 1.5×10^{12} particles, while one A_{260} unit of freshly purified, disaggregated virus of BTV1 SA or BTV10, contained 2.28×10^{12} and 2.52×10^{12} particles, respectively. One A_{260} unit of ISVP of BTV1 SA contained 2.15×10^{12} particles. Although not identical, these results provide reasonably good support for the particle numbers calculated using the biochemical data as described above, which are used throughout the paper.

Cells

KC cells were kindly provided by Sally Wechsler, USDA Laramie, and were grown in Schneiders insect media (Sigma) containing 5% foetal calf serum, at room temperature (15 to 29°). *A. albopictus* (C6/36) cells were provided by Colin Leake (London School of Hygiene and Tropical Medicine), were grown in RPMI 1640 media, modified by the addition of 20 m*M* HEPES buffer and containing 10% foetal calf serum at 29°. BHK21 cells were grown in Eagles media containing 5% foetal calf serum at 37°.

Infectivity analysis of BTV particles for BHK21, *A. albopictus* (C6/36), and *C. variipennis* (KC) cells

Serial 10-fold dilutions of virus, ISVP, and core particles were prepared and inoculated onto microtitre plates containing confluent monolayers of BHK21, KC, or C6/36 cells. The BHK cells were incubated at 37° and the KC and C6/36 cells at 29°. The titration end points were read on Day 7 p.i. and end point titres were calculated using the method of Spearmann and Karber (Finney, 1964). Viral CPE is difficult to distinguish in *A. albopictus* cells and was undetectable in KC cells (Wechsler and McHolland, 1988). In order to detect virus replication in the insect cells, at Day 7 p.i. 20 μl of the contents of each well of all of the titrations were transferred to duplicate microtitre plates containing confluent monolayers of BHK21 cells, and the results were determined by development of CPE after a further incubation for 1 week at 37°.

Infection of insects

Adult *C. nubeculosus* and *C. variipennis* females were infected with BTV using artificial feeding techniques (Mellor *et al.,* 1974). Flies were fed, when 3 to 4 days old, using the different BTV particles in bovine blood (oral infection). No cleavage of the VP2 present in the outer capsid layer of the virus particles was detected after incubation with bovine blood or serum, using the incubation conditions described by Marchi *et al.* (1995), or by Western blotting using antiserum to SDS–PAGE purified VP2 (results not shown). The infectivity of the feeding mixtures for BHK21 cells was assayed after each experiment. To ascertain the titre of virus present in each fly on Day 0, representative numbers of flies were assayed, either individually or in groups, as indicated. The remaining flies were held at $25° \pm 1°$ until required for further virus assays. Samples of flies were assayed either individually, or as pools, at intervals up to 240 hr p.i. Virus titrations were carried out in microtitre plates by inoculation of 10-fold dilution series of suspensions of titurated midges or feeding mixtures onto BHK21 cells (Mellor *et al.,* 1974). Virus concentrations were calculated using the methods of Spearman and Karber (Finney,

1964) and were expressed as BHK $\log_{10}$ $TCID_{50}$ per fly, or per milliliter.

The intact virus particles used for oral infectivity studies were maintained in the absence of the detergent NLS, which was used during their purification and to maintain them in a disaggregated state (Mertens *et al.,* 1987). NLS proved to be toxic to the flies, causing a high level of early mortality (up to 100% within 24 hr), significantly reduced the percentage of flies that would feed, and reduced the volume of blood meal ingested. Removal of the detergent caused aggregation of the majority of the intact virus particles (Mertens *et al.,* 1987) and reproducibly resulted in a reduction in specific infectivity of those virus particles which remained in suspension of approximately 20-fold. No significant aggregation of core particles or ISVP was observed under the storage conditions used (in the absence of detergent).

RESULTS

Protein composition of purified BTV particles used for infectivity studies

Intact virus particles, ISVP, and cores of BTV1 SA, BTV3, and BTV4 were purified as described by Mertens *et al.* (1987). The protein composition of these preparations was routinely checked by SDS–PAGE, followed by silver staining, staining with Coomassie blue, or fluorography of radiolabelled preparations. The protein compositions of typical preparations of the three different particle types of BTV1 SA, as used for the infectivity studies described here, are analysed in Fig. 1. This figure illustrates the presence of small amounts of NS2 consistently found on the outer capsid surface of the intact virions; the loss of NS2 and cleavage of VP2 in the outer capsid layer of ISVP; and the complete loss of outer capsid components, VP2, VP5 and NS2 from the core particles. The core is composed of an outer layer of VP7 trimers, surrounding a scaffolding of VP3, containing the minor core proteins VP1, VP4, VP6/VP6a, and the dsRNA genome segments (Verwoerd *et al.,* 1972; Mertens *et al.,* 1987; Hewat *et al.,* 1992; Grimes *et al.,* 1995). Apart from minor differences in electrophoretic migration, the protein composition of the different purified particles of BTV3 and BTV4 were directly comparable to those of BTV1 SA (data not shown).

Infection of insect and mammalian cell cultures

The time course of virus production in KC and BHK, cells infected with BTV ISA particles at a concentration of 10^6 particles/ml, was analysed by titration of tissue culture supernatants in BHK cells over a 48-hr period (Fig. 2). After an initial eclipse period of approximately 7 hr, the level of infectious virus produced in cells of either type infected with the intact disaggregated virus particles progressively increased up to 28 hr p.i. After this period

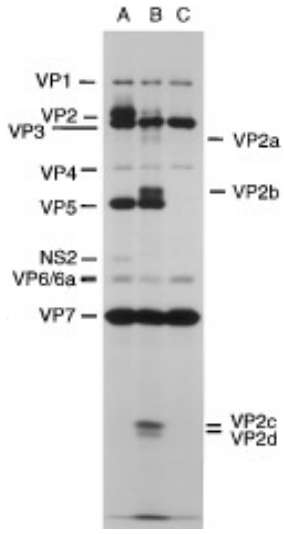

FIG. 1. Typical preparations of virus particles (lane A), ISVP (lane B), and cores (lane C) of BTV1 SA, labelled with [^{35}S]methionine, which were grown and purified as described by Mertens *et al.* (1987), were analysed by SDS–PAGE, using an 11% gel and a Laemmli (1970) buffer system. The protein bands were then detected by fluorography. The migration positions of the structural proteins, the minor amount of NS2 associated with the outer capsid layer of the intact virions, and the cleavage products of VP2 present in ISVP, which have previously been identified (Mertens *et al.,* 1984, 1987), are marked.

virus production in the BHK cell cultures reached a plateau of approximately 10^7 BHK $TCID_{50}$/ml; however, most of the cells had died and no further increase was detected. No CPE was observed in the infected KC cells and although the virus titre had only reached 10^5 BHK $TCID_{50}$/ml by 28 hr p.i., it continued to rise up to the end of the experiment at 48 hr p.i. By this time the virus titre had also reached approximately 10^7 BHK $TCID_{50}$/ml. In the KC and BHK cells infected with ISVP (at similar particle concentrations), the eclipse period was reduced to 5 or 3 hr, respectively. Core particles (at the same particle concentration) failed to infect the BHK cell cultures but did infect KC cells, with an eclipse period and virus production curve that were very similar to those produced with the intact virus particles.

The specific infectivities of the different particle types of BTV 1SA were assayed using two insect cell lines (from *C. variipennis* or *A. albopictus*) as well as mammalian cells (BHK 21 cells) (see Table 1). Some overall similarity was observed with the insect cells, with a 100- to 1000-fold higher specific infectivity associated with ISVP than with the disaggregated or aggregated virus particles, respectively, and 100-fold increase in the infectivity of ISVP for the insect cells, as compared to mammalian cells. The infectivity of ISVP for insect cells, calculated at 1.9×10^{11} $TCID_{50}/A_{260}$ unit (Table 1), is significantly higher than previous estimates for purified BTV particles in mammalian cells, or for intact virus in insect cells. (Verwoerd *et al.,* 1972; Wechsler and McHolland, 1988; Mertens *et al.,* 1987). We have calculated that this level

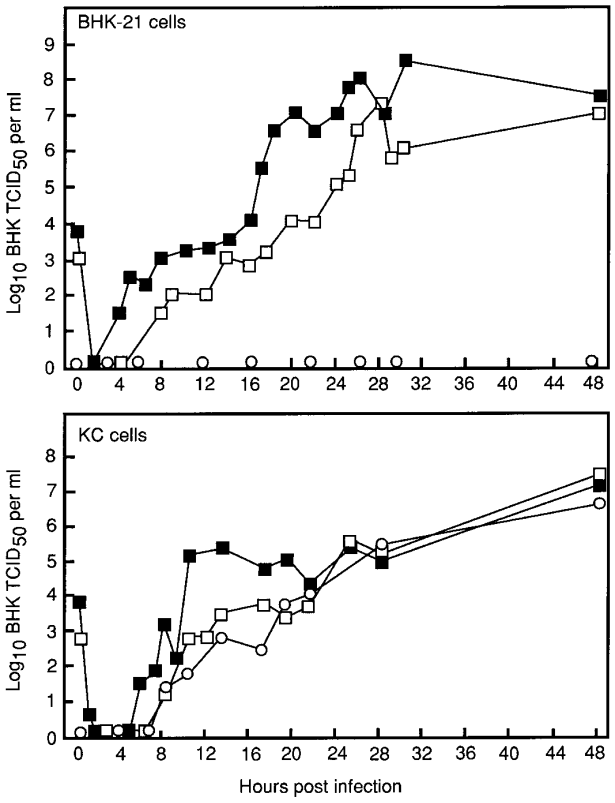

FIG. 2. Monolayers of BHK21 cells, or KC cells, were infected with purified virus particles (□), ISVP (■), or cores (○) of BTV1 SA at final concentrations of approximately 10^6 particles/ml. At the times indicated the tissue culture media from the infected cells were sampled and the amounts of infectious virus present measured by titration in BHK 21 cells. The KC cells were grown at 22° ± 2°.

of specific infectivity is equivalent to one insect cell $TCID_{50}$ per 13 particles (Table 1). The KC cells were also much more susceptible than either C6/36 or BHK cells to infection by cores (approximately 1000-fold relative increase in core infectivity). However, intact virus particles were found to have essentially the same specific infectivity for each of the cell lines tested and consistently showed an approximately 10-fold decrease in infectivity, thought to be due to aggregation, on removal of the NLS by dialysis or centrifugation. Comparisons of 12 different preparations of virus particles of BTV serotypes 1, 3, and 4 showed that removal of NLS caused a reduction in specific infectivity of those virus particles which remain in suspension of between 11- and 20-fold (Mertens, unpublished data; Mertens *et al.,* 1987). The specific infectivity of core particles for KC (but not for C6/36 cells) is directly comparable to that of the intact but aggregated virus particles. Due to the toxic nature of NLS for adult insects, these aggregated virus particle preparations were also used for oral infection studies.

No outer capsid proteins were detected in preparations of purified BTV core particles by SDS–PAGE, followed by silver staining or fluorography (Fig. 1). Recent studies have also shown that antibodies raised against "purified" core particles did not neutralise either ISVP or intact virions (Mertens *et al.,* 1995) and did not immunoprecipitate VP2 or VP5 (Wade-Evans *et al.,* 1988), indicating the absence of the serotype specific outer coat proteins that are involved in serum neutralisation. However, it was considered possible that core particles may be infectious for mammalian cells due to retention of very low levels of outer capsid proteins. Under these circumstances the higher infectivity of ISVP for the *Culicoides* cells could possibly help to explain the much higher infectivity for these cells also observed with core particles, which were usually made from ISVP (Mertens *et al.,* 1987). Cores were made by CsCl treatment during CsCl gradient centrifugation from intact virus of BTV1 SA and showed no significant difference in specific infectivity for KC cells to those made from ISVP (data not shown). This suggests that the infectivity associated with the purified core particles for *Culicoides* cells and probably also for the other insect and mammalian cells is not simply due to residual amounts of outer coat proteins.

Oral infection of *C. variipennis* and *C. nubeculosus* with BTV virus particles, ISVP, and cores

The yield of infectious virus and the time course of infection in whole vector insects produced by aggregated

TABLE 1

Infectivity of BTV1 SA Particles for Insect and Mammalian Tissue Culture

Particle type	$TCID_{50}$ per A_{260} unit[a] (particles per $TCID_{50}$)[b]		
	BHK cells	KC cells	C6/36 cells
Disaggregated[c] virus	2.4×10^9 (1.0×10^3)[b]	2.4×10^9 (1.0×10^3)[b]	2.5×10^9 (1.0×10^3)[b]
Aggregated[d] virus	1.9×10^8 (1.3×10^4)[b]	1.3×10^8 (1.9×10^4)[b]	1.3×10^8 (1.9×10^4)[b]
ISVP	2.1×10^9 (1.2×10^3)[b]	1.9×10^{11} (13)[b]	1.9×10^{11} (13)[b]
Cores[a]	7.1×10^4 (3.2×10^7)[b]	1.2×10^8 (1.9×10^4)[b]	1.5×10^5 (1.5×10^7)[b]

[a] The values for the specific infectivities in tissue culture that are given represent the results from several different experiments, each of which was repeated at least three times. The results showed variations of approximately twofold or less in each case, except with core particles, which proved to be relatively unstable during storage, resulting in a progressive reduction in their infectivity. The data presented are for freshly prepared particles in each case.

[b] The equivalent numbers of particles per $TCID_{50}$ are shown in parentheses.

[c] Disaggregated virus particle stored in 0.1 *M* Tris/HCl, pH 8.0, containing 0.1% sodium-*N*-lauroylsarcosine.

[d] Aggregated virus particles stored in 0.1 *M* Tris/HCl, pH 8.0.

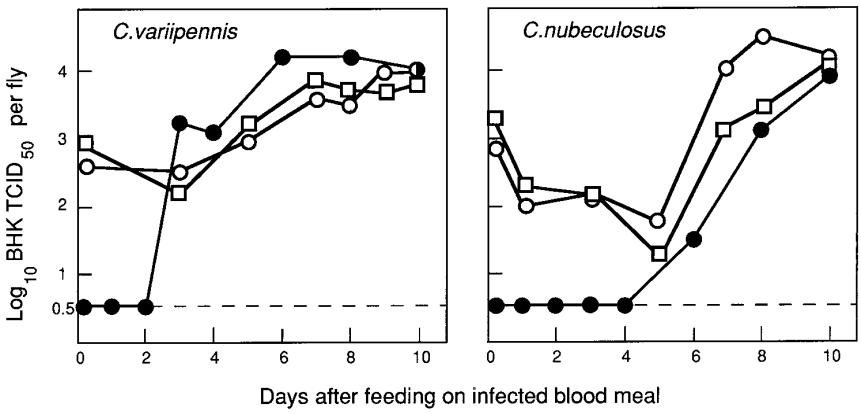

FIG. 3. Pools of 10 adult females of *C. variipennis* and *C. nubeculosus* were infected orally with purified virus particles (○), ISVP (□), or core particles (●) of BTV4. Each of the particle types had been purified by CsCl gradient centrifugation, dialysed against and then stored in 0.1 *M* Tris/HCl buffer, pH 8.0, in the absence of detergent. As previously reported this resulted in aggregation of the intact virus particles (Mertens *et al.,* 1987) and causes a decrease in the infectivity for cell cultures of those particles which remain in suspension, of approximately 1 $\log_{10}$, compared to disaggregated virus particles (see also Table 1). Particles were mixed with bovine blood at a final concentration of 3.2×10^{11} particles/ml ($10^{11.5}$ particles/ml) estimated from measurement of their optical density at 260 nm. This concentration of particles is equivalent to 2.5×10^7, 2.7×10^8, and 1.0×10^3 BHK $TCID_{50}$/ml for intact virus, ISVP, and cores, respectively (Table 1). These approximate levels of infectivity were confirmed by titration in BHK21 cells. After feeding, the pools of infected flies were assayed for infectious BTV at the times indicated. Infectivities $\leqslant 0.5$ Log_{10} BHK $TCID_{50}$/fly were not detectable in these assays. Similar results were obtained with purified particles of BTV1 SA and BTV3 (data not shown). The experiments described were carried out at least twice in each case, with similar results. The data presented are from a single experiment.

virus particles (without NLS), ISVP, or cores of BTV4, ingested as part of a blood meal, is shown in Fig. 3. The infectivity detected on Day 0, in pools of flies infected with either virus particles or ISVP (measured as $TCID_{50}$ for BHK21 cells (BHK $TCID_{50}$ per fly) is thought to be due to the presence of the ingested particles and declined over the first 3 to 5 days postinfection (in *C. variipennis* and *C. nubeculosus,* respectively). This reduction may have been due to complete particle degradation, or to loss of outer capsid proteins, thereby releasing the core particles which are much less infectious for BHK cells. The level of infectivity in pools of flies infected with core particles, over the same period, remained below detectable levels. From 2, or 4/5 days postinfection (in *C. variipennis* and *C. nubeculosus,* respectively), the titre of infectious virus present increased and had reached $\geqslant 10^4$ BHK $TCID_{50}$ per fly by Day 10. This represented an increase of at least 10-fold over the infectivity due to ingested particles on Day 0 and is taken as evidence of infection and virus replication. In experiments where particles were incorporated into blood meals at concentrations that were insufficient to initiate infection, some infectivity due to the ingested ISVP or virus, but not core particles, was detected on Day 0 (using BHK cells). However, by Day 7 or earlier this had sunk below detectable levels, where it remained until at least Day 10, indicating loss or degradation of particles and the absence of replication (data not shown). Results essentially identical to those shown in Fig. 3 were also obtained with purified particles of BTV1 SA and BTV3 (data not shown). In subsequent experiments unless otherwise stated flies were assayed for infection at Day 10.

Virus production after oral infection of pooled groups of 20 adult *C. variipennis* and *C. nubeculosus* with preparations of purified but aggregated virus particles, ISVP, or cores of BTV1 SA, at a range of different particle concentrations, is shown in Fig. 4. Ingestion of virus particles resulted in infection of at least one individual of *C. variipennis* in each pool at concentrations $\geqslant 9.0 \times 10^9$ particles/ml (Fig. 4, top) (equivalent to 6.9×10^5 BHK $TCID_{50}$/ml). However, any individuals within the pools of *C. nubeculosus* were only infected at approximately 25-fold higher concentrations of the intact virus ($\geqslant 2.2 \times 10^{11}$ particles/ml, equivalent to 1.7×10^7 BHK $TCID_{50}$/ml).

Infection was detected in the pools of flies from either insect species at core particle concentrations $\geqslant 1.6 \times 10^{10}$/ml (Fig. 4, bottom). As a result of the low infectivity of cores for mammalian cells, this was equivalent to only 500 BHK $TCID_{50}$/ml. In contrast to the results obtained using virus particles there was no difference detected in these initial experiments in the minimum amount of ingested core particles required to infect individuals of *C. nubeculosus* or *C. variipennis.*

Some of the pooled individuals of *C. variipennis* became infected at 2500-fold lower ISVP particle concentrations than with either virus or cores (at 3.5×10^6 particles/ml; 2.9×10^3 BHK $TCID_{50}$/ml) (Fig. 4 middle). Although the pooled *C. nubeculosus* also became infected at lower concentrations of ISVP (at 5×10^9 particles/ml; 2.9×10^6 BHK $TCID_{50}$/ml), the differences compared to cores or intact virus particles were much less marked, at 4-fold and 60-fold lower, respectively. The apparently greater similarity in specific infectivity of ISVP and virus/cores for *C. nubeculosus* may be in part due to the limita-

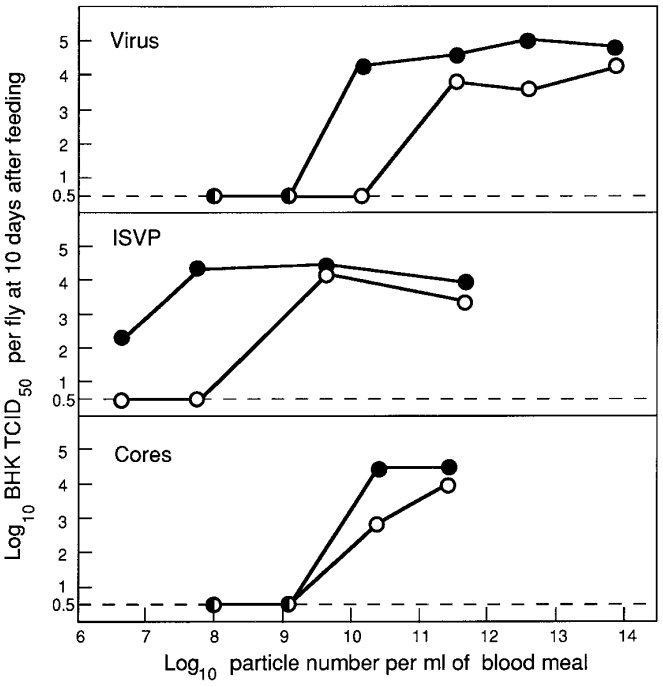

FIG. 4. In a series of experiments essentially similar to those illustrated in Fig. 3, the production of infectious virus in adult females of *C. variipennis* (●) and *C. nubeculosus* (○) was assayed at Day 10 post-oral-infection with BTV ISA. Groups of 20 flies were fed on bovine blood containing varying concentrations of virus particles (top), ISVP (middle), or cores (bottom). Cores and ISVP were dialysed against and stored in 0.1 *M* Tris/HCl, pH 8.0. The virus preparation, which as previously reported was partially aggregated in the absence of NLS (Mertens *et al.,* 1987) contained >40% w/v sucrose after purification. The production of infectious virus at Day 10, in $TCID_{50}$ per fly, was assayed for each pool of insects in BHK 21 cells. Infectious virus ⩽0.5 Log_{10} BHK $TCID_{50}$/fly was not detectable in these assays. Essentially similar results were obtained with particles of BTV3 and BTV4 and with unpurified intact virus particles of BTV1 SA (particle concentration calculated from BHK $TCID_{50}$/ml, assuming them to be disaggregated) (data not shown). These experiments were carried out at least twice in each case. The data presented are from a single experiment.

tions in the number of different particle concentrations assayed in these initial experiments. However, infection of at least one individual of *C. variipennis* was detected at 1000-fold lower ISVP concentrations than with *C. nubeculosus.*

In each case where oral infection was detected in a pool of flies, with the exception of two groups, the virus titre produced at Day 10, was in the range $10^{3.5}$–10^5 BHK $TCID_{50}$/fly. The two lower yields, one for each *Culicoides* species, were both at the lowest infectious concentration of particles (either ISVP or cores) and may indicate a single infected individual within the group, an infection that was still progressing to the higher levels, one in which the infection was restricted to gut cells (Mellor, 1990), or a combination of these factors. Data essentially similar to those presented in Fig. 4 were obtained with preparations of purified particles of BTV3 and BTV4 and with unpurified virus particles of BTV1 SA (tissue culture supernatant, with particle concentration calculated from BHK $TCID_{50}$ per milliliter. In these experiments, the minimumum particle concentrations at which infection of the pools of adult flies were detected were within twofold of those shown in Fig. 4, in each case, except with ISVP, where no infected individuals were detected below 2×10^7 particles/ml (five times higher than with BTVISA ISVP in *C. variipennis* in Fig. 4).

Figure 5 shows the percentage of individual *C. variipennis* and *C. nubeculosus* flies that were infected orally by different BTV particle types when ingested at a range of particle concentrations. The aggregated virus particles and cores produced relatively similar infection rate curves in *C. variipennis* (Fig. 5, bottom), while cores were required at approximately five times lower concentrations than virus particles to produce approximately the same proportion of infected individuals of *C. nubeculosus* (Fig. 5, top). With both insect species ISVP were found to have a significantly higher specific infectivity than either aggregated virus particles or cores (approximately 100-fold higher for *C. variipennis,* and 100-fold higher than virus particles or 20-fold higher than cores, respectively, for *C. nubeculosus*). Approximately 40 times higher concentrations of ISVP or aggregated virus particles were required to produce the same percentage of infected flies of *C. nubeculosus* than with *C. variipennis.* However, only 5 times more particles were required with the prepa-

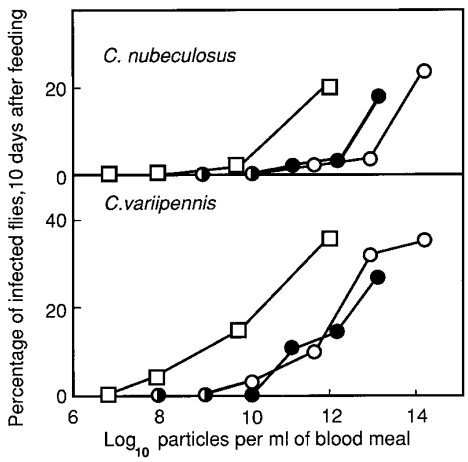

FIG. 5. In experiments essentially similar to those described in the legends to Figs. 3 and 4, adult females of *C. nubeculosus* (top) and *C. variipennis* (bottom) were fed on bovine blood containing varying amounts of purified BTV ISA virus particles (○), ISVP (□), and cores (●). ISVP and core preparations were stored in 0.1 *M* Tris/HCl buffer, pH 8.0, virus particle preparation also contained >40% sucrose. Groups of 30–40 flies were assayed individually for the presence of infectious BTV at Day 10 postinfection by titration in BHK cells. Data are presented from a single experiment. Similar results to those shown (up to maximum concentration of 10^{12} particles/ml) were obtained with purified virus preparations, from which the sucrose had been removed by dialysis, with ISVP preparations containing sucrose and with unpurified BTV1 SA (tissue culture supernatant, using particle concentration calculated from BHK $TCID_{50}$/ml, assuming a disaggregated state; data not shown).

TABLE 2

The Effect of Protease Inhibitors on Infection of *C. variipennis* by Infectious Subviral Particles or Virus Particles of BTV1 SA

Composition of blood meal	Number of flies fed on Day 0	% of flies infected at 10 days postfeeding (infection rate)	Change in % infection rate caused by protease inhibitor addition
Whole blood + Virus particles[a]	50	40	—
Whole blood + Virus particles[a] + Protease inhibitors[b]	25	8	Minus 32%
Whole blood + ISVP[a]	100	38	—
Whole blood + ISVP[a] + Protease inhibitors[b]	20	45	Plus 7%

[a] Virus particles or ISVP were fed to individuals of *C. variipennis* at 7.3 or 7.5 Log_{10} BHK cell $TCID_{50}$/ml, respectively.

[b] Protease inhibitors were added as indicated and contained 1 mg/ml final concentration of Bowman–Birk inhibitor (trypsin–chymotrypsin inhibitor, Sigma) and 1000 units/ml final concentrates of aprotinin (Bayer).

rations of cores and the lowest concentration of cores at which infection was observed was the same in both species (10^{11} particles/ml).

In Fig. 5, the minimum concentration of ISVP of BTV1 SA to cause infection in *C. variipennis* was 7.0×10^7 particles/ml, which is 20-fold higher than that observed in Fig. 4 (3.5×10^6 particles/ml). This difference could reflect some variation in the susceptibility of different batches of adult insects to BTV infection, the infection of only a single insect at this concentration of ISVP in Fig. 4 (see above), and the unavoidable limitations imposed by using a finite number of different particle concentrations in these studies.

The lowest concentrations of purified particles at which any individuals flies became infected in Figs. 4 and 5 were used to estimate the minimum number of core, ISVP, or aggregated virus particles required to infect each of the two vector species via the oral route (Table 3). Despite the limited number of particle concentrations assayed, there appears to be some correlation between the specific infectivity of the different particles for KC cells and the minimum insect infectious dose (MIID), in terms of the particle numbers at which infection of individuals of *C. variipennis* occurred (Tables 1 and 3), with a 25- to 100-fold higher particle number required in each case. However, there was little or no apparent correlation between the specific infectivities of the different particles for KC cells and the observed MIID for individuals of *C. nubeculosus,* with the higher concentrations required ranging from 100- to 25,000-fold.

The effect of protease inhibitors on infection of *C. variipennis* by purified BTV particles

Proteases within the lumen of the insect gut could cause the conversion of ingested BTV virus particles to ISVP. In order to analyse the significance of any such conversion during infection of the whole insect, virus particles and ISVP of BTV1 SA were fed to groups of *C. variipennis* in the presence or absence of protease inhibitors (Table 2). It was found that the presence of inhibitors reduced the percentage of individual flies in-

TABLE 3

Oral Infectivity of BTV1 SA Particles for Adult *Culicoides*

Particle type	Minimum insect infectious doses per A_{260} Unit[a,b] (particles per minimum insect infectious dose)[a,c]	
	C. variipennis	*C. nubeculosus*
Aggregated[d] virus	2.8×10^6 (9.0×10^5)[c]	1.1×10^5 (2.2×10^7)[c]
ISVP	7.2×10^9 (350)[c]	7.2×10^6 (3.5×10^5)[c]
Cores[b]	1.6×10^6 (1.2×10^6)[c]	1.6×10^6 (1.2×10^6)[c]

[a] Assuming a typical blood meal of 0.1 μl (Mellor, 1990), to calculate the smallest number of particles which infected any individual flies in Figs. 3 and 4.

[b] Core particles proved to be relatively unstable during storage, resulting in a progressive reduction in their infectivity for cell cultures. The data presented are therefore for freshly prepared particles in each case.

[c] The equivalent numbers of particles per minimum insect infectious dose are shown in brackets.

[d] Disaggregated virus particle containing 0.1% sodium-*N*-lauroylsarcosine were not used for oral infection studies because the detergent proved to be poisonous for the flies and significantly reduced the percentage that would feed. Aggregated virus particles were stored in 0.1 *M* Tris/HCl, pH 8.0

fected by the whole virus particles, administered as part of a blood meal, by a factor of 5 but had no comparable effect on infection rates with ISVP, where a small increase in the percentage of infected flies, (by a factor of approximately 0.2), was observed.

DISCUSSION

Analysis of the infectivity of the different BTV particle types using *C. variipennis* and *A. albopictus* cell lines demonstrated that ISVP are approximately 100-fold more infectious for these insect cells than disaggregated intact virus particles and were 1000-fold more infectious than the preparations of aggregated virus (from which NLS had been removed), which were also used for oral infection studies in adult *Culicoides.* Although these results confirm that the intact virus particles are infectious in their own right for insect cells, conversion of virus to the more infectious ISVP (or any change in the conversion rate) could be an important factor in the efficiency of infection of insect cells.

A large relative increase in the specific infectivity ($\times 10^3$) of the BTV core was also observed in the *Culicoides* cell cultures, as compared to mammalian (BHK 21) or mosquito cells. The difference in core particle infectivity for KC and C6/36 cells suggests some fundamental differences in these different insect cells and possibly in the insects from which they were derived, only one of which (*C. variipennis*) is a vector for BTV.

The reasons for the relatively high infectivity associated with core particles in *Culicoides* cell systems are not known but could reflect some role of cores in the normal cell entry process. It has been shown that low pH can release the outer coat proteins of BTV to generate core particles (Verwoerd *et al.,* 1972; Huismans *et al.,* 1983). The low pH environment in endosomes may therefore cause conversion of virus particles to cores, prior to any contact with lysosomal proteases. Eaton *et al.* (1990) have reported that during uptake of BTV particles they leave endosomes prior to fusion with lysosomes. Core particle infectivity could therefore reflect an ability to cross the endosomal membrane and enter the cell cytoplasm, as has previously been suggested for ISVP of reovirus (Borsa *et al.,* 1979; Tosteson *et al.,* 1993) but without the involvement of outer capsid proteins. The higher infectivity of cores for *Culicoides* cells could reflect an increased rate of uptake into endosomes in these cells compared to the mosquito or mammalian cells.

Initiation of infection by intact BTV virus particles is thought to involve the interaction of outer capsid components with specific cell surface proteins or receptors, resulting in binding and entry into the host cells (Eaton *et al.,* 1990). Since the outer capsid layer has been removed from core particles, their infectivity cannot be due to conversion to either of the other particle types tested. The high level of core particle infectivity for KC cells suggests that the initial stages of BTV core/cell interaction and entry may use different receptors or mechanisms to those used by ISVP or virus particles and may indicate cell surface interactions with an outer core protein (e.g., VP7). These conclusions are supported by the observation that the specific infectivities of BTV core preparations for individuals of the different insect species tested were mutually closer than were those of either ISVP or virus particles (Fig. 4, Table 3). However, the mechanism by which cores of BTV initiate infection of KC cells does not appear to be so effective in mammalian cells and may even reflect a reduction or absence of suitable receptors/entry mechanisms.

The different BTV particle types have specific infectivities, which appear to vary independently in the three cell systems tested. A shorter eclipse period was also observed with ISVP but not cores, suggesting that all three particle types may have different cell attachment or entry mechanisms. It is possible that ISVP enter the cell via a direct membrane penetration route rather than via endosomes, as suggested for the equivalent particle of reovirus (Borsa *et al.,* 1979; Zarbl and Millward, 1983; Tosteson *et al.,* 1993). Previous cell binding studies have indicated that all three BTV particle types will bind to BHK and C6/36 cells (D. M. Jennings and P. P. C. Mertens, unpublished data). Studies of the role of core proteins in cell surface interactions, which are currently in progress, indicate that antibodies to the outer core protein VP7 can neutralise core particles but not intact virus or ISVP of BTV (Mertens *et al.,* manuscript in preparation), confirming the role of this protein in initiation of infection by cores.

Recent studies have demonstrated that the KC cells are nonproductively infected at a low level with a defective BTV (W. C. Wilson, personal communication). However, no virus that was infectious for BHK cells was generated in the uninoculated cells. In our studies we failed to detect BTV antigens by ELISA in uninoculated KC cells and no evidence for reassortment with superinfecting BTV was observed.

Due to its toxic nature for adult *Culicoides,* removal of the detergent NLS from purified virus preparations was essential prior to their use in oral infectivity studies in these insects. The aggregated nature of the virus particles used in these studies makes it more difficult to draw firm conclusions on the specific infectivity of the intact virus in whole insects, relative to that of either ISVP or cores. However, the studies described here, as well as earlier studies with BTV serotypes 1 and 4, demonstrated that removal of NLS from virus particle preparations consistently caused an approximately 20-fold drop in the specific infectivity of those particles that remained in suspension, for either mammalian or insect cell culture systems. When administered orally, purified but aggregated virus particles, ISVP, and cores of BTV serotypes 1, 3, and 4 can all infect some individuals of both insect species. The unchanged infectivities of the intact disaggre-

gated or aggregated virions for the three different cell lines and the higher infectivity of cores for the *Culicoides* cells appears to explain the similar infection rates observed with the cores and whole but aggregated virus particles for the insect vectors. In these studies ISVP were also found to be significantly more infectious than the intact but aggregated virus particles, producing the same percentage of infected flies at approximately 100-fold lower particle concentrations. This difference could be partially explained by the aggregated state of the virus particle preparations used. However, a smaller difference between the infectivity of the aggregated virus and ISVP was observed in whole insects, as compared to insect cells (100-fold as compared to 1000-fold lower). It is likely that the insect gut lumen will contain active proteolytic enzymes (Houseman, 1980; Champlain and Fisk, 1956; Akov, 1972; Gooding, 1972; Thomas and Gooding, 1972; Spiro-Kern and Chen, 1972). It was therefore considered possible that at least some of the ingested virus particles would be converted to ISVP by their action. Conversion of virus to ISVP by insect gut proteases could therefore have raised the overall infectivity of ingested virus particles by approximately 10-fold. The reduction in the infection rate produced by ingestion of intact virus but not ISVP caused by addition of protease inhibitors to the blood meal also indicates that proteolytic modification of virus particles may play a significant role in the normal infection processes involving intact virions, within vector insects. These observations suggest that 1 to 10% of the ingested intact virus particles were converted to ISVP by gut proteases. Any increase in the rate or percentage conversion of virus to ISVP could significantly increase the sensitivity of the cells lining the gut wall and consequently of a susceptible insect species, to initial infection by BTV.

Studies with African horse sickness virus (AHSV) demonstrated that serum proteases from some mammalian host species (in particular horses and dogs but not cows) resulted in up to 100% cleavage of VP2 on the outer surface of purified virus particles and significantly increased the infection rate of *C. variipennis* in oral feeding studies with unpurified virus (tissue culture supernatant) (Marchi *et al.,* 1995). However, no cleavage of the VP2 present in purified virus particles of BTV1 SA was detected on incubation with bovine, ovine, caprine, or porcine blood, or serum, under comparable reaction conditions (Burroughs, unpublished data). Bovine blood was mixed with the different particle preparations used for the oral infectivity studies described here. Bovine serum was also used in the mammalian and insect cell cultures used to grow and titrate the different BTV samples. The high level and species-specific nature of the VP2 cleavage that was observed with AHSV indicate that the conversion of whole virus particles to ISVP by host serum proteases may also be a significant factor in the susceptibility of flies to infection and could even influence the vector status of flies feeding on viraemic individuals of different mammalian species.

Although both *Culicoides* species can act as insect vectors of BTV, *C. nubeculosus* is less efficient in this respect than *C. variipennis* (Jennings and Mellor, 1988). Higher concentrations of ISVP and to a lesser extent virus particles, were required to infect the same percentage of individuals of *C. nubeculosus* compared to *C. variipennis* and some evidence for a longer eclipse period was observed (Fig. 3). The levels of peak BTV viraemia detected in blood from infected cattle or sheep have been estimated as 2×10^6 ELD_{50}/ml (Luedke *et al.,* 1967; Bowen *et al.,* 1985), or 2.5×10^4 and 4.0×10^5 BHK $TCID_{50}$/ml for cattle and sheep, respectively (Jeggo *et al.,* 1983; Roeder *et al.,* 1991). The failure to infect *C. nubeculosus,* by an oral route, at whole virus particle concentrations equivalent to or below 7.6×10^5 BHK $TCID_{50}$/ml of blood meal, may help to explain their lesser ability to act as a BTV vector (Mellor and Jennings, 1988) particularly in situations involving transmission from infected cattle. Some individuals of *C. variipennis* were infected at virus particle concentrations of $\geqslant 6.9 \times 10^5$ BHK $TCID_{50}$/ml of blood meal. From the studies presented here complete conversion of intact virus to ISVP by either host serum or insect gut proteases could raise the infectivity for *C. variipennis* by as much as 200- to 2000-fold, depending on the original level of particle aggregation in blood. Even the lower increase would effectively bring the level of peak viraemia in both sheep and cattle well above the minimum required for infection of some individuals of this vector species. However, in *C. nubeculosus* complete conversion of virus to ISVP might only raise BTV particle infectivity by as much as 6- to 60-fold (Table 1), even the higher level of increase, in this case would place the level of peak viraemia in cattle below the minimum and the level of viraemia in sheep only just above the minimum, at which we detected oral infection in this species (1.7×10^7 BHK $TCID_{50}$/ml). Even assuming that circulating intact virus has a reduced infectivity due to aggregation, this increase would only be sufficient to permit infection of some individuals of *C. nubeculosus* by feeding on sheep at the peak of viraemia. These observations suggest that any host, vector, or virus-specific factors which influence the efficiency of conversion of intact virus to ISVP will significantly affect the ability of *Culicoides* species to act as vectors for BTV. The higher level and greater difference observed in the susceptibility of the two species tested to infection by ISVP, compared to either virus or cores, suggest that such variability could also influence the vector competence of the species or even of individual flies. Larger numbers of individual flies of *C. variipennis* than *C. nubeculosus* became infected at each of the particle concentrations used in the oral infection studies, irrespective of particle type, suggesting that some factors involved in infection efficiency may also be independent of particle type or conversion.

The higher infectivity observed with ISVP of BTV indicates that conversion of virus particles by treatment with chymotrypsin followed by inoculation onto insect cells may provide an alternative and possibly more sensitive method for the isolation of orbiviruses from blood or other tissue samples, particularly in comparison to mammalian isolation systems (Mertens *et al.,* manuscript in preparation). The larger volumes that can be used in insect cell cultures suggest that such systems would also be more sensitive than direct inoculation or feeding of whole insects. Such improved isolation techniques may alter our current view of the height and duration of viraemia in infected animals, as has already been indicated by use of PCR (Katz *et al.,* 1994; MacLachlan *et al.,* 1994) and may have implications for our current models of the epidemiology of these viruses (Lehane, 1981; Lord, unpublished data). Studies are in progress to reevaluate the duration of detectable viraemia after BTV infection in cattle by these techniques.

ACKNOWLEDGMENTS

The authors thank Dr. M. Jennings for useful discussion, Mr. E. P. Denison for production of adult *Culicoides,* Mr. G. S. Pero for production of tissue culture cells and media, Mrs. C. Chisholm for preparation of the manuscript, and Mr. B. Clarke for preparation of figures. This work was supported by the Ministry of Agriculture, Fisheries and Food and by European Community contract (African horse sickness in Europe: Grant No. 8001-CT91-0211).

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